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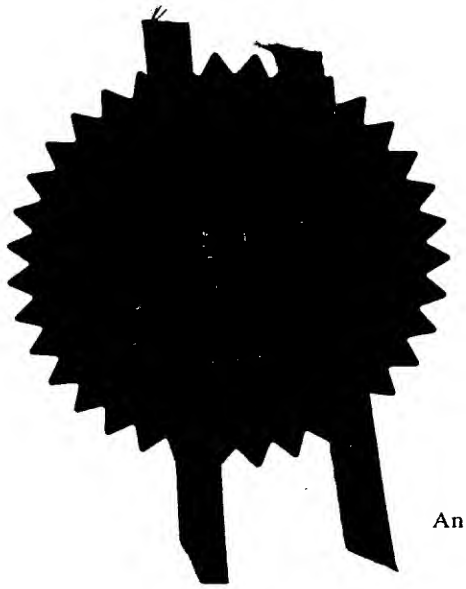

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4. Title of the invention
MONOCLONAL ANTIBODIES SPECIFIC FOR CYP1B1

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Monoclonal antibodies specific for CYP1B1

Cytochrome P450 (P450) CYP1B1 is the only known member of a recently identified sub-family of the CYP1 gene family. Human CYP1B1 was originally isolated from a dioxin treated keratinocyte cell line (Sutter et al., 1994) as part of a series of investigations to identify differential expression of genes caused by exposure to dioxin. The human CYP1B1 gene is located on chromosome 2p22-21 spanning 12kb and is composed of three exons and two introns (Tang et al., 1996). The mRNA is 5.2kb and encodes for a protein of 543 amino acids (Sutter et al., 1994). This is the largest known human P450 gene both in terms of mRNA size and number of amino acids and is also the simplest structurally. Both nucleic acid and amino acid sequence analysis shows that CYP1B1 displays only approximately 40% homology with CYP1A1 and CYP1A2. The low degree of similarity with existing members of the CYP1 family resulted in this P450 being assigned to a new CYP1 sub-family CYP1B which to date only contains the single member CYP1B1. Indeed hybridisation studies of human DNA suggests that there is only one member of the CYP1B gene family (Sutter et al., 1994). The CYP1B1 gene is transcriptionally activated by ligands of the Ah receptor including planar aromatic hydrocarbons (Sutter et al., 1994, Hakkola et al., 1997) and the most potent of these Ah receptor agonists for inducing transcription of the CYP1B1 gene appears to be dioxin (Hakkola et al., 1997).

Orthologous forms of this P450 have also recently been isolated from a benzanthracene-induced cell line derived from mouse embryo fibroblasts (Savas et al., 1994; Shen et al., 1994) and adult rat adrenal cortex (Bhattacharyya et al., 1995; Walker et al., 1995). Although there is a high degree (greater than 80%) of both nucleic acid and amino acid sequence homology between the human, mouse and rat forms of CYP1B1 there also appears to be considerable species differences regarding tissue specific expression, regulation and metabolic specificity of CYP1B1 (Savas et al, 1994; Sutter et al., 1994; Bhattacharyya et al., 1995; Savas et al., 1997).

Breast cancer is the commonest cancer to affect women and is an estrogen dependent tumour. Human CYP1B1 expressed in yeast (S. cerevisiae) shows high specific activity towards the 4-hydroxylation of 17β-estradiol (Hayes et al., 1996) converting it to 4-hydroxyestradiol and indeed human CYP1B1 is considered to be the most efficient 4-hydroxylase of 17β-estradiol. In contrast mouse CYP1B1 does not appear to act as an estradiol hydroxylase (Savas et al., 1997) indicating species differences in the metabolic capability of CYP1B1. Liehr and Ricci (1996) showed that there is a significant level of 4-hydroxylation of 17β-estradiol in breast cancer microsomes. They showed considerably higher 4-hydroxylation of 17β-estradiol in microsomes prepared from breast cancer compared with only a very low level of 4-hydroxylation present in normal breast tissue. Both immunoreactive CYP1B1 protein (Murray et al., 1997) and CYP1B1 mRNA (McKay et al., 1995) have been identified in breast cancer indicating that CYP1B1 is a major form of cytochrome P450 expressed in breast cancer.

Our initial immunohistochemical studies of CYP1B1 (Murray et al., 1997) which showed enhanced CYP1B1 expression in several types of human cancer including breast cancer were performed using a polyclonal antibody to CYP1B1. Here we have developed monoclonal antibodies specific for human CYP1B1 using a synthetic peptide as the immunogen, characterised the antibodies for use in immunohistochemistry and used these antibodies to investigate the expression of CYP1B1 by immunohistochemistry in a series of primary human breast cancers.

P450 sequence alignment, peptide selection and immunisation

Based on a combination of structural homology modelling and sequence alignment of the human CYP1B1 amino acid sequence with the human CYP1A1 and CYP1A2 amino acid sequences a 148 amino acid segment located in the C-terminal third of the CYP1B1 protein was

predicted to contain regions of amino acids which would be located on the external aspect of the CYP1B1 protein. Peptides of either 14 or 15 amino acid residues corresponding to this segment of the CYP1B1 protein were synthesised in the University of Aberdeen Protein Facility. The individual peptide sequences and amino acid location on the CYP1B1 protein are listed in table 1. Individual peptides were then conjugated to ovalbumin using glutaraldehyde as previously described (Duncan et al., 1992) and each peptide conjugate used as an immunogen. Individual peptide conjugates mixed with Freund's incomplete adjuvant were injected *i.p* into BALB/c mice and the mice were re-immunised with the same peptide conjugate 2-4 weeks following the initial immunisation. The presence of an immune response to each peptide conjugate was determined by testing serum (obtained 10 days after the second immunisation) from each mouse for recognition of expressed CYP1B1 by immunoblotting. The mice whose sera gave the best recognition of CYP1B1 were then given a final immunisation with the appropriate peptide conjugate and used for the production of monoclonal antibodies.

Monoclonal antibodies to CYP1B1

Four days after the final immunisation with peptide conjugate the mice were sacrificed, their spleens isolated and splenic cells fused with mouse myeloma cells (Ag8.653). The resultant hybridoma clones were then screened for antibody production by enzyme linked immunoassay (ELISA) using the relevant peptide conjugated with bovine serum albumin (BSA). The BSA conjugates were bound to an ELISA plate by incubation overnight at 4°C in 50mM sodium carbonate/bicarbonate buffer, pH 9.6, and the ELISA was performed as described previously (Duncan et al., 1992, Murray et al., 1998b). Hybridomas clones which were strongly positive by ELISA were subcloned twice and further tested by immunoblotting using expressed CYP1B1, expressed CYP1A1, control microsomes and human liver microsomes. The monoclonal antibodies were isotyped using an Isostrip kit (Roche Diagnostics, Lewes, Sussex, UK) which was performed according to the manufacturer's instructions.

Tissues

Samples of normal tissues (liver, kidney, lung, pancreas, adrenal cortex, brain, stomach, jejunum, colon, breast and ovary) were obtained from fresh, unfixed tissue samples submitted to the Department of Pathology, University of Aberdeen for diagnosis. Samples of tissue were frozen in liquid nitrogen and stored at -75°C prior to preparation of microsomes. Samples of primary breast cancer (n=61) were obtained from samples of breast tissue submitted to the Department of Pathology, University of Aberdeen for diagnosis. All the samples of breast tissue were from needle core biopsies of palpable breast lumps performed as the diagnostic protocol prior to definitive treatment. The core biopsies were fixed in 10% neutral buffered formalin at room temperature and then routinely embedded in wax.

The diagnosis of breast cancer was performed with haematoxylin and eosin stained sections using standard histopathological criteria. The tumours were graded according to criteria described by Elston and Ellis (1991) and the estrogen receptor status of the breast cancers was assessed by immunohistochemistry for oestrogen receptor protein as previously described (King et al., 1997). The lymph node status was assessed from subsequent axillary lymph node samples submitted for histopathological examination. The clinico-pathological characteristics of the breast cancers are described in Table 2.

Expressed CYP1B1 and CYP1A1

Microsomes prepared from human lymphoblastoid cells containing expressed human CYP1B1, expressed human CYP1A1 or control lymphoblastoid cells which only contained vector were obtained from Gentest Corp. Woburn, Ma.

Preparation of microsomes

Frozen samples of tissues were thawed on ice in 0.01M Tris-HCl pH 7.4 containing 1.15% KCl. The thawed samples of tissue were dissected free of connective tissue and fat, finely chopped using a scalpel and then homogenised in 0.01M Tris-HCl containing 0.25M sucrose and 15% glycerol using a Polytron PT3000 homogeniser (Kinematica AG, Switzerland). The homogenates were then centrifuged at 15,000g for twenty minutes at 4°C using a Centrikon T-124 centrifuge (Kontron Instruments, Cumbernauld, UK). The resultant supernatants were then centrifuged at 180,000g (44,000rpm) for one hour at 4°C using a Centrikon T-1160 centrifuge (Kontron Instruments). The pellet obtained after centrifugation was resuspended in 0.1M Tris-HCl containing 15% glycerol and 1mM EDTA (Sigma-Aldrich Co. Poole, Dorset, UK) and centrifuged again at 180,000g (44,000rpm) for one hour at 4°C. The final microsomal pellet was re-suspended in 0.1M Tris-HCl containing 15% glycerol and 1mM EDTA and the microsomal samples were then stored at -75°C prior to use. The protein concentration of each sample of microsomes was determined using Bradford's method (Bradford, 1976). Bovine serum albumin (Sigma-Aldrich) was used as the protein standard.

Immunoblotting

Samples of microsomal proteins were electrophoretically separated at constant current in a 10% polyacrylamide gel using a Hoefer SE600 vertical gel electrophoresis apparatus (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) and then transferred at constant current for 18 hours to nitrocellulose (Hybond ECL, Amersham Pharmacia Biotech) by electroblotting using a Hoefer TE42 blotting system (Amersham Pharmacia Biotech). After electrophoretic transfer non-specific protein binding sites were blocked by incubation of the nitrocellulose membrane for 60 minutes at room temperature in wash buffer consisting of 2% non-fat milk (Marvel, Premier Beverages, Stafford, UK) in 10mM phosphate buffered saline containing 0.05% Tween 20 (Sigma). The nitrocellulose was then sequentially incubated with immune mouse serum (1/250) or CYP1B1 monoclonal antibody (1/100) and goat anti-mouse immunoglobulin conjugated to horseradish peroxidase (1/2000; Bio-Rad, Hemel Hempstead, UK). Following incubation with each antibody the membrane was washed for five 10 minute periods with wash buffer and after removal of unbound secondary antibody the membrane was further washed in 10mM phosphate buffered saline for five 10 minute periods. Horseradish peroxidase was then demonstrated using an enhanced chemiluminescent technique (ECL plus, Amersham Pharmacia Biotech) which was performed as previously described (McKay et al., 1995; Murray et al., 1997). Briefly the nitrocellulose membrane was incubated in a solution consisting of 4ml of detection reagent 1 (lumigen PS-3, Amersham Pharmacia Biotech) and 100µl of detection solution 2 (luminol PS-3, Amersham Pharmacia Biotech) for 5 minutes at room temperature, blotted dry, wrapped in clear plastic film and then exposed to X-ray film (Amersham Pharmacia Biotech).

Immunohistochemistry

Immunohistochemical detection of CYP1B1 was performed using a catalysed signal amplification method (King et al., 1997). In this study we used fluorescein tyramide rather than biotinylated tyramide which we had used in our previous study (King et al., 1997) to avoid any possible interference from endogenous biotin. Sections (4µm in thickness) were cut onto aminoethyl propoxy silane coated slides then dewaxed in xylene, rehydrated in 100% ethanol, 95% ethanol and washed in 0.05M Tris-HCl pH7.6 containing 150mM NaCl (TBS). Endogenous peroxidase was inhibited using a solution consisting of 90ml methanol and 3ml hydrogen peroxide. In some experiments antigen retrieval was performed by microwaving the sections in 0.01M citrate buffer pH6.0 for 20 minutes in microwave (Proline™, Proline, UK) operated at full power (800W) while in other experiments no antigen retrieval was performed. After the antigen retrieval step sections were then allowed to cool to room temperature and then the primary monoclonal anti-CYP1B1 antibody applied.

The primary antibody was applied as tissue culture supernatant at various dilutions (undiluted to 1/160) for 60 minutes at room temperature. After incubation in primary antibody the sections were washed in TBS for three successive 5 minute periods and then peroxidase conjugated rabbit anti-mouse immunoglobulin (1/100 in TBS containing 4% normal human serum, Dako, High Wycombe, UK) was applied for 30 minutes at room temperature. The sections were then washed in TBS and TBS containing 0.05% Tween 20 (TNT buffer). The sections were then further washed in TNT buffer and fluorescein tyramide (NEN, Hounslow, Middlesex, UK) applied for 10 minutes at room temperature. The sections were then further washed in TNT buffer followed by a application of monoclonal mouse anti-fluorescein (1/20, Dako) for 30 minutes at room temperature. Following further washing in TNT buffer peroxidase conjugated rabbit anti-mouse immunoglobulin (1/100 in TBS containing 4% normal human serum) was applied for 30 minutes at room temperature. After washing in TBS sites of bound peroxidase were then demonstrated colorimetrically using a solution containing diaminobenzidine and hydrogen peroxide (Liquid DAB plus, Dako). After incubating the sections for 10 minutes at room temperature in peroxidase substrate solution, the reaction was stopped by washing the slides in cold tap water and the enzyme reaction product was intensified using 0.5% copper sulphate. The slides were then washed in cold tap water, counterstained with haematoxylin, dehydrated in alcohol, cleared in xylene and mounted in a synthetic mounting media (DPX, BDH, Poole, Dorset, UK). The sections were examined using bright field light microscopy by two independent observers (MCEM, GIM) in order to establish the presence or absence of immunostaining, and its distribution and localisation and intensity. A tumour was regarded as positive if any tumour cells showed immunostaining while a tumour was classified as negative if there was a complete absence of immunostaining in tumour cells.

Positive control tissue was sections of a breast cancer which we have previously shown to contain CYP1B1 by both immunoblotting and immunohistochemistry with a polyclonal antibody to CYP1B1 (Murray et al., 1997). Negative controls used in place of the primary monoclonal antibody were TBS and tissue culture media . In one experiment antibody was liquid phase pre-absorbed with the peptide (peptide E, 10nmol of peptide/ml antibody) which had been used as the immunogen for the monoclonal antibodies prior to performing immunohistochemistry.

Development of monoclonal antibodies to CYP1B1

The immune response of sera from mice injected with each of the peptide conjugates was assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis and immunoblotting using expressed CYP1B1 as the antigen. The sera of mice injected with different peptides showed a variable immune response (figure 1). Sera of mice which had been injected with peptides D and E both showed recognition of CYP1B1 and were judged to have a produced a positive immune response. Sera from mice injected with peptide E gave a marginally stronger recognition of expressed human CYP1B1 than peptide D. However, sera from mice injected with peptides A and B and peptides F to J showed no apparent recognition of CYP1B1 and were considered to have produced no significant immune response. Mice which had been immunised with peptide E were therefore chosen to develop monoclonal antibodies to CYP1B1. The peptide sequence used to develop the monoclonal antibodies shows a high degree of similarity with corresponding rat CYP1B1 and mouse CYP1B1 sequences with 13 of the 15 amino acid residues being identical (figure 2).

Five monoclonal antibodies were developed from mice immunised with peptide E. The individual antibodies were designated 5C4, 5D3, 5D9, 5E2 and 5G7 and isotyping showed that all the antibodies are IgG1κ subtype. All the monoclonal antibodies recognised a single immunoreactive band of molecular size 52kDa corresponding to the expected molecular size of expressed human CYP1B1 by immunoblotting and did not recognise expressed human CYP1A1, or any protein present in vector only control microsomes or human liver microsomes (figure 3). Serial dilutions of expressed CYP1B1 indicated that the minimum detectable amount of CYP1B1 by immunoblotting was 0.05 pmol of expressed CYP1B1 (figure 4).

CYP1B1 was not identified by immunoblotting microsomes prepared from a range of normal adult human tissues including kidney, stomach, small intestine, colon and lung (figure 5).

Immunohistochemistry on formalin fixed wax embedded sections of breast cancer used as the positive control showed that three of the monoclonal antibodies (5D3, 5E2 and 5G7) demonstrated strong staining while two of the antibodies (5C4, 5D9) showed no immunoreactivity. All the monoclonal antibodies which showed positive immunoreactivity required an antigen retrieval step for optimum immunohistochemical results and all three antibodies showed an identical pattern of localisation and distribution of immunohistochemical staining. Therefore only one of the monoclonal antibodies (5D3) was used for the subsequent immunohistochemical studies of breast cancer. Liquid phase pre-incubation of anti-CYP1B1 antibody with peptide E prior to performing immunohistochemistry almost completely abolished immunoreactivity.

CYP1B1 immunoreactivity was identified in 47 (77%) of cases of breast cancer while there was no detectable CYP1B1 immunoreactivity in 14 cases (23 %). In each case in which there was CYP1B1 immunoreactivity the immunoreactivity was localised to the cytoplasm of tumour cells. The intensity of the immunoreactivity ranged from strong in 10 (16.4%) cases, moderate in 12 (19.7%) cases and weak immunoreactivity was present in 25 (41%) cases. The presence of CYP1B1 in different grades, histological types of breast cancer and lymph node status is summarised in tables 3 - 5. There was no CYP1B1 immunoreactivity in stromal cells or connective tissue nor in the following cell types including lymphocytes and plasma cells when they were present in individual biopsies. The presence of CYP1B1 was associated with the presence of estrogen receptor protein (table 6, $\chi^2 = 8.54$; $p = 0.03$) while there was no relationship between the presence of CYP1B1 and the histological type of the tumour, tumour grade or the presence or absence of lymph node metastasis.

CYP1B1 shows increased expression in a variety of tumours including breast cancer (Murray et al., 1997) and there is elevated CYP1B1 associated 4-estradiol hydroxylase activity in breast cancer (Liehr and Ricci et al., 1996). CYP1B1 is also capable of metabolizing a variety of putative human carcinogens including polycyclic aromatic hydrocarbons and heterocyclic amines (Shimada et al., 1996; Crespi et al., 1997). Thus CYP1B1 appears to have potentially important roles in tumour development and progression, as a potential target for anti-cancer drugs and as a tumour biomarker. Our initial studies of the presence of CYP1B1 in individual types of tumours were performed with a polyclonal antibody to CYP1B1 and for each tumour type only a small number of tumour samples were investigated (Murray et al., 1997). To evaluate more fully the potential of CYP1B1 (tumour development and progression) as a tumour marker requires monoclonal antibodies that specifically recognise CYP1B1 in formalin fixed wax embedded tissue sections and also requires a larger number of tumors to be studied. In this study we have developed monoclonal antibodies to CYP1B1, demonstrated that they sensitively and specifically detect CYP1B1 by immunoblotting and immunohistochemistry and then used these antibodies to investigate the presence of CYP1B1 in a series of primary breast cancers.

The strategy we used to develop monoclonal antibodies to CYP1B1 was a combination of structural molecular modelling and sequence alignment to identify regions of CYP1B1 which were likely to be located on the external aspect of the CYP1B1 protein and thus likely to be immunogenic. We have recently used a similar approach to develop monoclonal antibodies to matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases (Murray et al., 1998a and b). A region located in the C-terminal third of the CYP1B1 protein encompassing the haem binding region was identified and peptides consisting of either 14 or 15 amino acid residues were synthesised and conjugated to carrier protein. Each peptide was injected into mice and the immunoreactivity of each peptide for CYP1B1 was assessed by immunoblotting using microsomes prepared from human lymphoblastoid cells containing expressed CYP1B1 and microsomes containing vector only. Only sera from mice immunised with peptides D and E showed recognition of CYP1B1 whereas none of the other peptides showed significant immunoreactivity for CYP1B1. Mice which had been immunised with peptide E were selected for the development of monoclonal antibodies to CYP1B1. The peptide sequence used for the generation of the monoclonal antibodies shows a high degree of similarity (13 out of 15 amino

acids identical) with rat CYP1B1 and mouse CYP1B1 and it would be anticipated that the monoclonal antibodies would also recognise rat CYP1B1 and mouse CYP1B1. All the monoclonal antibodies were specific for CYP1B1 and did not recognise either CYP1A1 or CYP1A2 the other known members of the CYP1 gene family. Furthermore the antibodies did not recognise any other protein in human liver microsomes. Human liver microsomes were used as a source for CYP1A2 as CYP1A2 is one of the major forms of P450 constitutively expressed in liver and liver microsomes also acted as a source for several other forms of P450 thus providing a broad screen to confirm the specificity of the antibodies for CYP1B1.

In this study we were not able to detect CYP1B1 by immunoblotting in a range of normal human tissues. The absence of CYP1B1 protein in both normal human liver and a range of extra-hepatic tissues is consistent with our previous immunohistochemical studies (Murray et al., 1997) in that we were unable to detect CYP1B1 protein. In this study we loaded a relatively high amount of microsomal protein (30μg) per lane and used a highly sensitive chemiluminescent detection system so it seems likely that even a very low level of CYP1B1 in the normal tissues studied would have been detected using this system.

A further major aim of this study was to develop antibodies to CYP1B1 which could be used in immunohistochemistry and that were effective on formalin fixed wax embedded sections of tissue. All the monoclonal antibodies were evaluated by immunohistochemistry using formalin fixed wax embedded sections of breast cancer which we have previously shown by immunoblotting to contain a relatively high level CYP1B1 (Murray et al., 1997) and we found that three of the monoclonal antibodies effectively detected CYP1B1 by immunohistochemistry. Since the three antibodies that gave positive immunoreactivity all produced an identical pattern and intensity of immunoreactivity we used only one of the antibodies to investigate CYP1B1 expression.

In this study we found that 77% of breast cancers contained CYP1B1 and in each tumor CYP1B1 was specifically localised to tumor cells. The high frequency of expression of CYP1B1 in breast cancer is very similar to our previous studies of a small number of breast cancers and would support the concept that CYP1B1 is a major form of cytochrome P450 present in breast cancer (McKay et al., 1995; Murray et al., 1997). CYP1B1 was found in all histological types of breast cancer and the presence of CYP1B1 was not associated with any particular histological type of breast cancer nor the presence or absence of lymph node metastases, however the expression of CYP1B1 did correlate with the presence of estrogen receptor protein. This is of interest as recently an association between a CYP1B1 polymorphism (valine/leucine) at amino acid 432 and estrogen receptor status in breast cancer has been described (Bailey et al., 1998). There is also the potential for "cross talk" between the estrogen receptor complex and the aryl hydrocarbon receptor complex (Wang et al., 1998) which is involved in the transcriptional regulation of CYP1B1 (Schmidt and Bradfield, 1996).

Since CYP1B1 is involved in estrogen metabolism acting as a specific C4 hydroxylase of estradiol (Hayes et al., 1996) then the presence of CYP1B1 in breast cancer cells is likely to make a significant contribution to the intra-tumoral metabolism of estradiol. The presence of CYP1B1 in breast cancer also provides a molecular target for drugs specifically activated by CYP1B1.

The development of monoclonal antibodies to CYP1B1 that are effective in formalin fixed wax embedded sections should make them useful for investigating CYP1B1 expression in different tumour types and related pre-neoplastic lesions. The antiodies can e used for the diagnosis of cancer. The antibodies can also be developed as the basis of immunochemical in vivo diagnostic tests and as therapeutics targeted to the CYP1B1 protein or a degradation product thereof.

The role of CYP1B1 in tumour cells is unknown. However, the capability of CYP1B1 in metabolism of several currently used antitumour drugs means that inhibition of CYP1B1 activity in tumours is likely to improve the efficacy of such drugs.

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Table 1

Peptide sequences and amino acid location on CYP1B1 protein of peptides used for immunisation

Peptide	Peptide sequence	Location of peptide in CYP1B1 protein
A	NLPYVLAFLYEAMRF	377-391
B	SSFVPVTIPHATTAN	392-406
C	TSVLGYHIPKDTVVF	407-421
D	VNQWSVNHDPVKWPN	422-436
E	PENFDPARFLDKDGL	437-451
F	INKDLTSRVMIFSVG	452-466
G	KRRCIGEELSKMQLF	467-481
H	LFISILAHQCDFRAN	482-496
I	PNEPAKMNFSYGLT	497-510
J	IKPKSFKVNVTLRE	511-524

Table 2

Clinico-pathological characteristics of breast cancer

Age (mean and range)	52.3 years (34-76)
Histological type	
Invasive ductal carcinoma	52 (85.2%)
Invasive lobular carcinoma	8 (13.1%)
Tubulo-lobular carcinoma	1 (1.7%)
Grade of breast cancer	
Grade 1	10 (16.4%)
Grade 2	27 (44.3%)
Grade 3	24 (39.3%)
Lymph node status	
negative (no metastasis)	34 (55.7%)
positive (presence of metastasis)	22 (36.1%)
not determined	5 (8.2%)
Estrogen receptor status	
negative	25 (41%)
positive	34 (55.7%)
not determined	2 (3.3%)

Table 3

The presence of CYP1B1 in different histological types of breast cancer

	Histological type of breast cancer		
	Invasive Ductal (n=52)	Invasive lobular (n=8)	Tubulo-lobular (n=1)
CYP1B1 immunoreactivity			
Negative (n=14)	12	2	0
Weak (n=25)	20	4	1
Moderate (n=12)	11	1	0
Strong (n=10)	9	1	0

Table 4

Comparison of CYP1B1 with different grades of breast cancer

	Grade of breast cancer		
	Grade 1 (n=10)	Grade 2 (n=27)	Grade 3 (n=24)
CYP1B1 immunoreactivity			
Negative (n=14)	2	6	6
Weak (n=25)	4	12	9
Moderate (n=12)	3	5	4
Strong (n=10)	1	4	5

Table 5

Comparison of CYP1B1 in lymph node positive and lymph node negative breast cancer cases

	Lymph node status	
	Lymph node positive (n=34)	Lymph node negative (n=22)
CYP1B1 immunoreactivity		
Negative (n=14)	6	8
Weak (n=24)	16	8
Moderate (n=11)	6	5
Strong (n=7)	6	1

Table 6

Correlation of the presence of CYP1B1 immunoreactivity in breast cancer with the presence of estrogen receptor (χ^2 =8.54; p = 0.03)

	Estrogen receptor status	
	negative (n=25)	positive (n=34)
CYP1B1 immunoreactivity		
Negative (n=13)	2	11
Weak (n=25)	14	11
Moderate (n=12)	7	5
Strong (n=9)	2	7

Figure Legends

Figure 1

Region of CYP1B1 protein to which individual peptides were synthesised and used for immunisation of mice. The immunoreactivity of individual mouse sera towards CYP1B1 following immunisation with each peptide is indicated.

Figure 2

Sequence alignment of the peptide (peptide E) used to generate the monoclonal antibodies to CYP1B1 with the corresponding sequences of mouse and rat CYP1B1. Amino acid residues common to all three P450s are indicated with an asterisk.

Figure 3

Immunoblot of expressed CYP1B1 with monoclonal antibody (5D3) demonstrating specificity for CYP1B1. Lane 1, expressed CYP1B1 (10μg of microsomal protein, 0.86 pmol of CYP1B1); lane 2, expressed CYP1A1 (10μg of microsomal protein, 0.53 pmol of CYP1A1); lane 3, control lymphoblastoid microsomes containing only vector (10μg of microsomal protein) and lane 4, normal human liver microsomes (10μg of microsomal protein). Molecular weight markers are shown on the right in kiloDaltons.

Figure 4

Immunoblot demonstrating the minimum detectable amount of CYP1B1 using monoclonal antibody 5D3. Lane 1 to lane 6 decreasing amounts of expressed CYP1B1. Lane 1, 0.86 pmol of CYP1B1; lane 2, 0.43 pmol of CYP1B1; lane 3, 0.21 pmol of CYP1B1; lane 4, 0.1 pmol CYP1B1; lane 5, 0.05 pmol of CYP1B1 and lane 6, 0.025pmol of CYP1B1. Molecular weight markers are shown on the right in kiloDaltons.

Figure 5

Immunoblot of CYP1B1 of microsomes prepared from various normal human tissues. Lane 1, expressed CYP1B1 (10μg of microsomal protein corresponding to 0.86pmol of CYP1B1); lane 2, liver; lane 3, kidney; lane 4, lung; lane 5, pancreas; lane 6, adrenal cortex; lane 7, brain (medulla); lane 8, stomach; lane 9, jejunum; lane 10, colon; lane 11, breast and lane 12, ovary (30μg of microsomal protein loaded per lane of normal human tissue). Molecular weight markers are shown on the right in kiloDaltons.

Figure 6

Immunohistochemical demonstration of CYP1B1 in breast cancer using monoclonal antibody 5D3, A. Localisation of CYP1B1 in a grade 3 invasive ductal carcinoma. There is strong immunoreactivity for CYP1B1 in tumor cells, B. localisation of CYP1B1 in an invasive lobular carcinoma demonstrating strong immunoreactivity in breast cancer cells and C. There is no immunoreactivity in a grade 3 invasive ductal carcinoma when the primary CYP1B1 monoclonal antibody is replaced by TBS in the immunohistochemical procedure. Scale bar represents 60 μm.

Fig 1

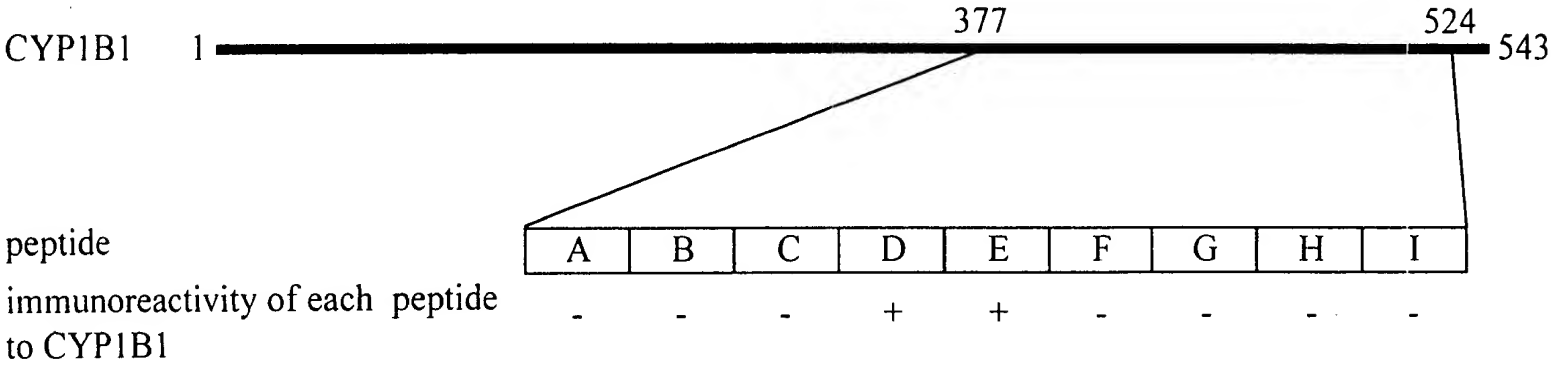
CYP1B1
1
377
524
543
peptide
A
B
C
D
E
F
G
H
I
immunoreactivity of each peptide to CYP1B1
-
-
-
+
+
-
-
-
-

FIG 2

```
human CYP1B1    PENFDPARFLDKDGL      (peptide E)
rat CYP1B1      PEDFDPARFLDKDGF
mouse CYP1B1    PEDFDPARFLDKDGF
                ** ***********
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Fig 3

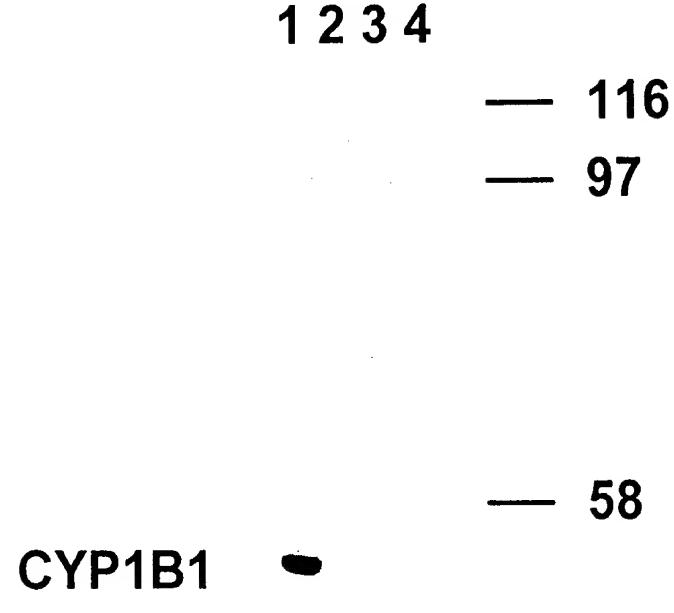


Fig 4

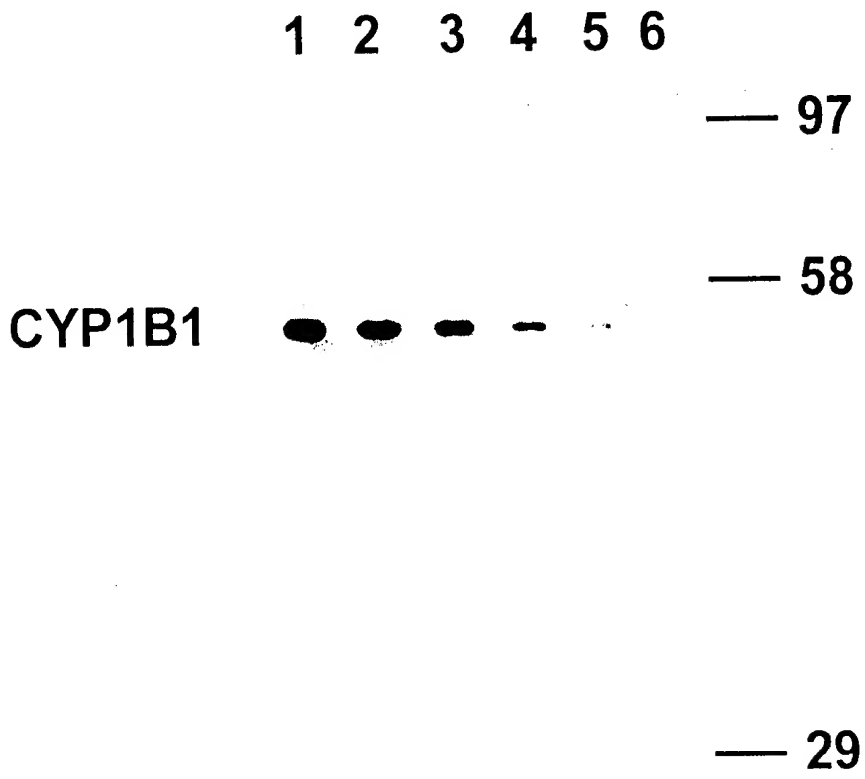


Fig 5

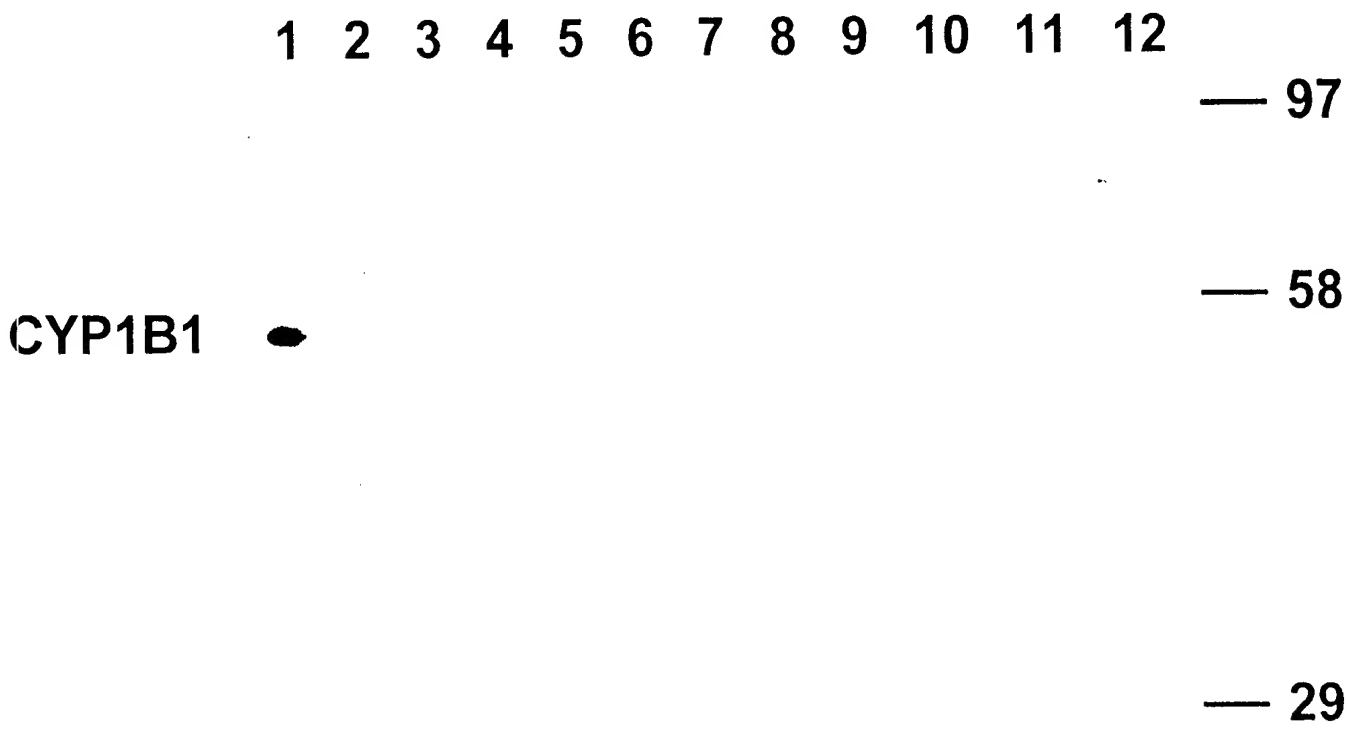
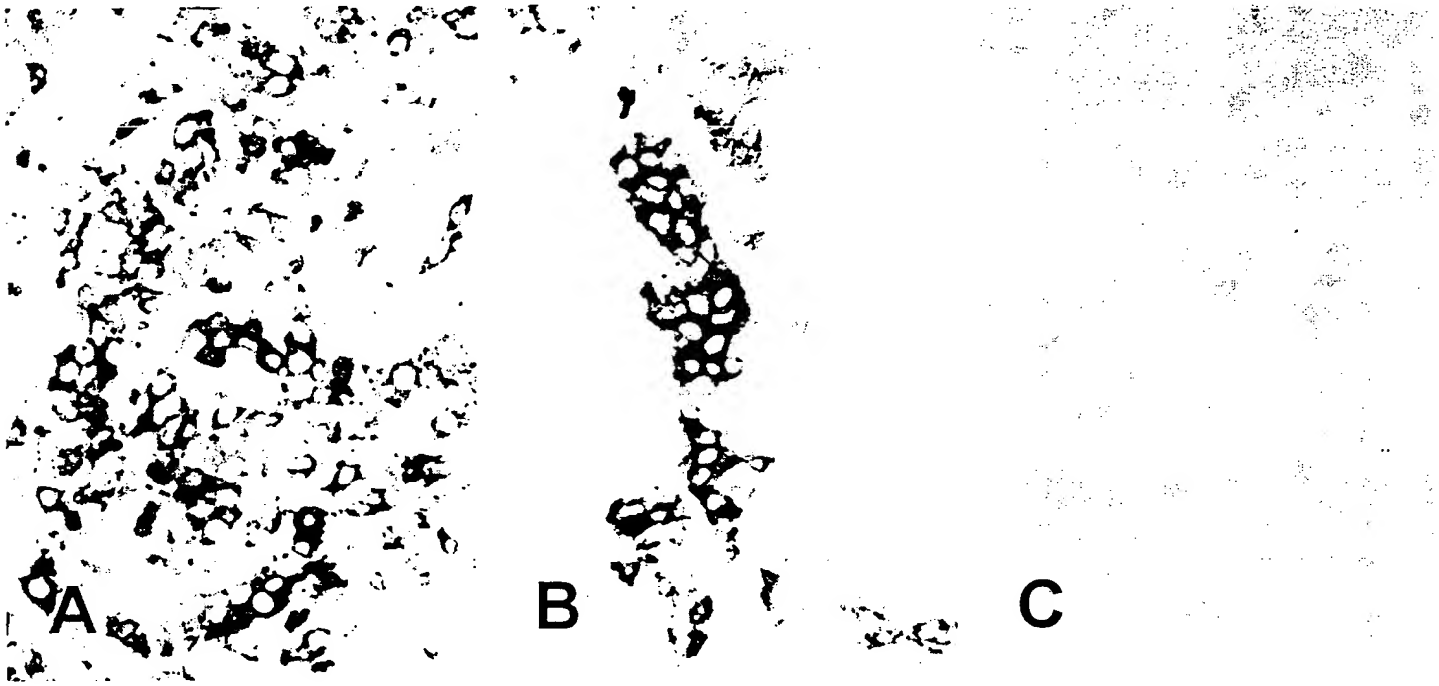


Fig 6



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